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Short communication

# Dinitrophenol modulates gene expression levels of angiogenic, cell survival and cardiomyogenic factors in bone marrow derived mesenchymal stem cells

Anwar Ali, Muhammad Aleem Akhter, Kanwal Haneef, Irfan Khan, Nadia Naeem, Rakhshinda Habib, Nurul Kabir, Asmat Salim *

*Dr. Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological Sciences, University of Karachi, Karachi-75270, Pakistan*

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ABSTRACT

Various preconditioning strategies influence regeneration properties of stem cells. Preconditioned stem cells generally show better cell survival, increased differentiation, enhanced paracrine effects, and improved homing to the injury site by regulating the expression of tissue-protective cytokines and growth factors. In this study, we analyzed gene expression pattern of growth factors through RT-PCR after treatment of mesenchymal stem cells (MSCs) with a metabolic inhibitor, 2,4 dinitrophenol (DNP) and subsequent re-oxygenation for periods of 2, 6, 12 and 24 h. These growth factors play important roles in cardiomyogenesis, angiogenesis and cell survival. Mixed pattern of gene expression was observed depending on the period of re-oxygenation. Of the 13 genes analyzed, ankyrin repeat domain 1 (Ankrd1) and GATA6 were downregulated after DNP treatment and subsequent re-oxygenations. Ankrd1 expression was, however, increased after 24 h of re-oxygenation. Placental growth factor (Pgf), endoglin (Eng), neuropilin (Nrp1) and jagged 1 (Jag1) were up-regulated after DNP treatment. Gradual increase was observed as re-oxygenation advances and by the end of the re-oxygenation period the expression started to decrease and ultimately regained normal values. Epiregulin (Ereg) was not expressed in normal MSCs but its expression increased gradually from 2 to 24 h after re-oxygenation. No change was observed in the expression level of connective tissue growth factor (Ctgf) at any time period after re-oxygenation. Kindlin3, kinase insert domain receptor (Kdr), myogenin (Myog), Tbx20 and endothelial tyrosine kinase (Tek) were not expressed either in normal cells or cells treated with DNP. It can be concluded from the present study that MSCs adjust their gene expression levels under the influence of DNP induced metabolic stress. Their levels of expression vary with varying re-oxygenation periods. Preconditioning of MSCs with DNP can be used for enhancing the potential of these cells for better regeneration.



## 1. Introduction

Stem cell therapy for the repair of damaged tissues has become the most prominent part of the current research in the field of regenerative medicine. Bone marrow derived mesenchymal stem cells (MSCs) are multipotent in nature and hence, have the potential to differentiate into various cell types including muscle, bone, neurons and endothelial cells along with the mesenchymal cell lineage (Salem and Thiemermann, 2010). The use of stem cell therapy has now become a promising option for the treatment of a variety of diseases including those of the cardiovascular system (Krishnan et al., 2006).

The mechanism of stem cell differentiation is still not fully understood. One way to understand the mechanism of stem cell mediated cellular regeneration is to identify the gene expression patterns of those factors that are involved in cell growth and differentiation process during normal development as well as during tissue repair. A number of research groups have analyzed the expression levels of different growth factors in MSCs under various cellular conditions. Gnecchi et al. (2008) have analyzed a number of growth factors which are important for survival and maintenance of pluripotency and those that take part in the process of cell adhesion, homing and proliferation during the process of trans-differentiation.

The poor viability of engrafted stem cells is a major concern for stem cell researchers. Stem cell death can be avoided by utilizing preconditioning strategies in which cells are exposed to non-cytotoxic stress or shock before transplantation into harsh ischemic microenvironment of the heart (Ma et al., 2009; Rosova et al., 2008). It has been

*Abbreviations:* MSCs, mesenchymal stem cells; DNP, 2,4 dinitrophenol; Ankrd1, ankyrin repeat domain 1; Pgf, placental growth factor; Eng, endoglin; Nrp1, neuropilin; Jag1, jagged 1; Ereg, epiregulin; Ctgf, connective tissue growth factor; Kdr, kinase insert domain receptor; Myog, myogenin; Tek, endothelial tyrosine kinase.

* Corresponding author.
*E-mail address:* asmat.salim@iccs.edu (A. Salim).

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0378-1119/

**Table 1**
Details of genes and their primers used in the study.

| No. | Genes | Functions | Primer sequences | Annealing Temp. (°C) | Product sizes |
|---|---|---|---|---|---|
| 1 | Ankyrin repeat domain 1 (Ankrd1) | Cardio-regulator | F: AGCGGAGCAACCAGCTATAA R: AAGTCTTGCTCCCCCAAAT | 51 | 214 |
| 2 | Connective tissue growth factor (Ctgf) | Tissue growth | F: TAGCAAGAGCTGGGTGTGTG R: TTCACTTGCCACAAGCTGTC | 58 | 156 |
| 3 | Endoglin (Eng) | Endothelial growth | F: TGCAGAAAGAGTCGGTTGTG R: TCTCAGTGCCATTTTGCTTG | 58 | 203 |
| 4 | Epiregulin (Ereg) | Epidermal growth | F: TCTGACATGGACGGCTACTG R: TCACGGTCAATGCAACGTAT | 59 | 163 |
| 5 | GATA binding factor 6 (Gata6) | Cardiomyogenesis | F: AACTGTGGCTCCATCCAGAC R: CATATAAAGCCCGCAAGCAT | 53 | 243 |
| 6 | Jagged 1 (Jag1) | Angiogenesis | F: AATGGGTGGAAAGGGAAAAC R: TGCAGACACAGGTGAAGGAG | 56 | 229 |
| 7 | Fermitin family member 3 (Kindlin3) | Cell adhesion | F: TGACCCAGCTGTATGAGCAG R: ATGGTGGTGAGGCTATCCAG | 59 | 244 |
| 8 | Kinase insert domain receptor (Kdr) | Angiogenesis | F: CCAAGCTCAGCACACAAAAA R: CCAACCACTCTGGGAACTGT | 58 | 190 |
| 9 | Myogenin (Myog) | Muscle development | F: TTTTTCATGCGACTCACAGC R: CTGTGGGAAAGAGTGGGTGT | 62 | 211 |
| 10 | Neuropilin (Nrp1) | Cell survival/angiogenesis | F: GGAGCTACTGGGCTGTGAAG R: ACCGTATGTCGGGAACTCTG | 58 | 208 |
| 11 | Placental growth factor (Pgf) | Growth/angiogenesis | F: AGGGTCATTGGACACCTGAG R: GGTCCTTCAAGGCAAAATCA | 59 | 230 |
| 12 | T-box 20 (Tbx20) | Early heart development | F: TCTGCAGAGGAGATCCGATT R: CCGGGAACTCAAAACTGTGT | 59 | 181 |
| 13 | Tyrosine kinase, endothelial (Tek) | Angiogenesis | F: GTGGGAAGTGGCAAAGTTGT R: TTCGGCATCAGACACAAGAG | 58 | 206 |

GAPDH was used as internal standard.
Primers: F: GAAAAGCTGTGGCGTGATGG and R: GTAGGCCATGAGGTCCACCA; annealing temperature: 60 °C and product size: 414 bp.

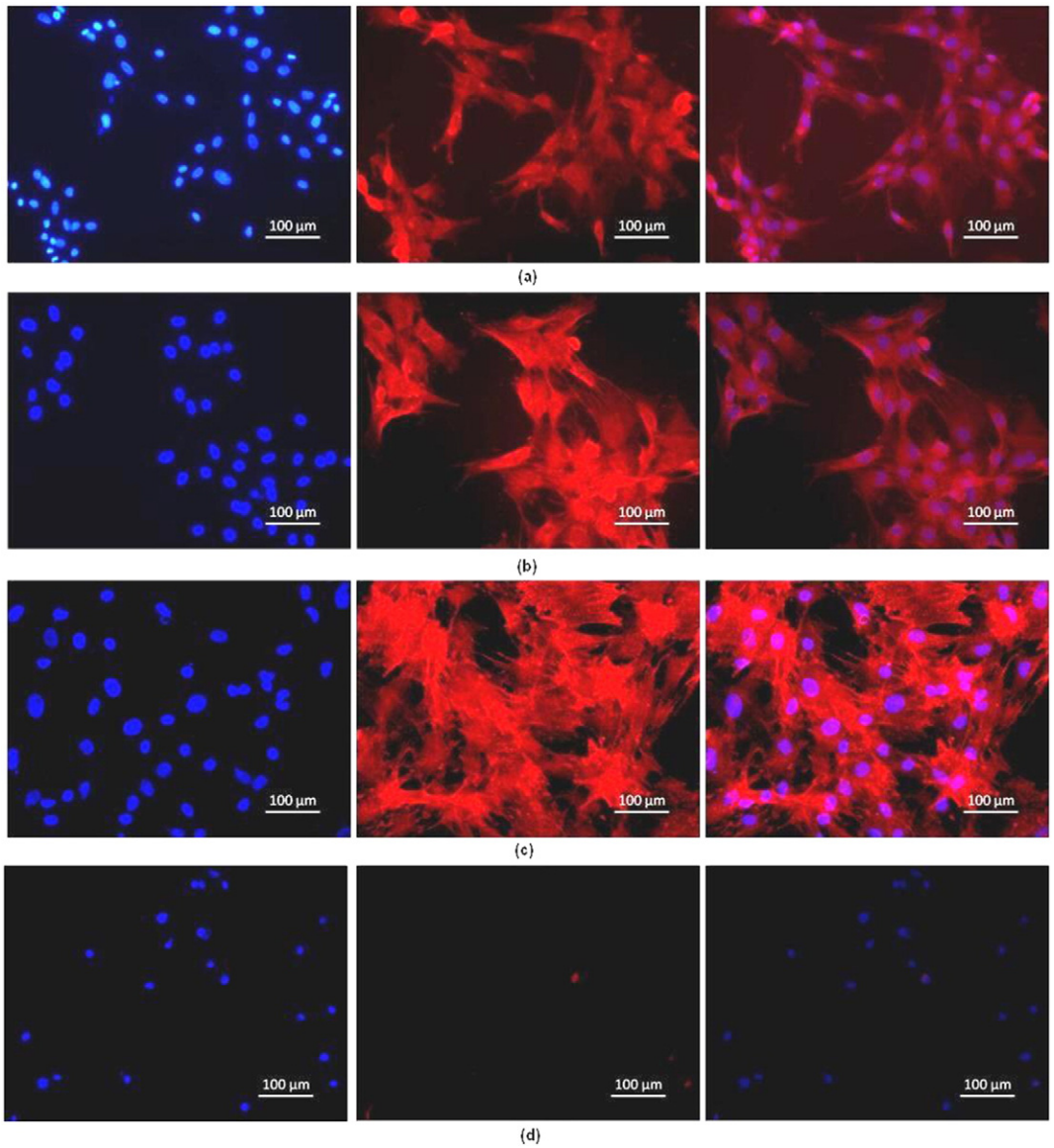


**Fig. 1.** Immuno-cytochemical analysis of mesenchymal stem cells (MSCs) for the presence of (a) c-kit, (b) CD44, (c) CD90 and (d) CD34 cell surface antigens. Alexa fluor 546 goat anti-mouse antibody was used as the secondary antibody. Nuclei were stained with DAPI. MSCs have shown positive expression of c-kit, CD44, and CD90 while CD34 which is a hematopoietic marker was not expressed in these cells.

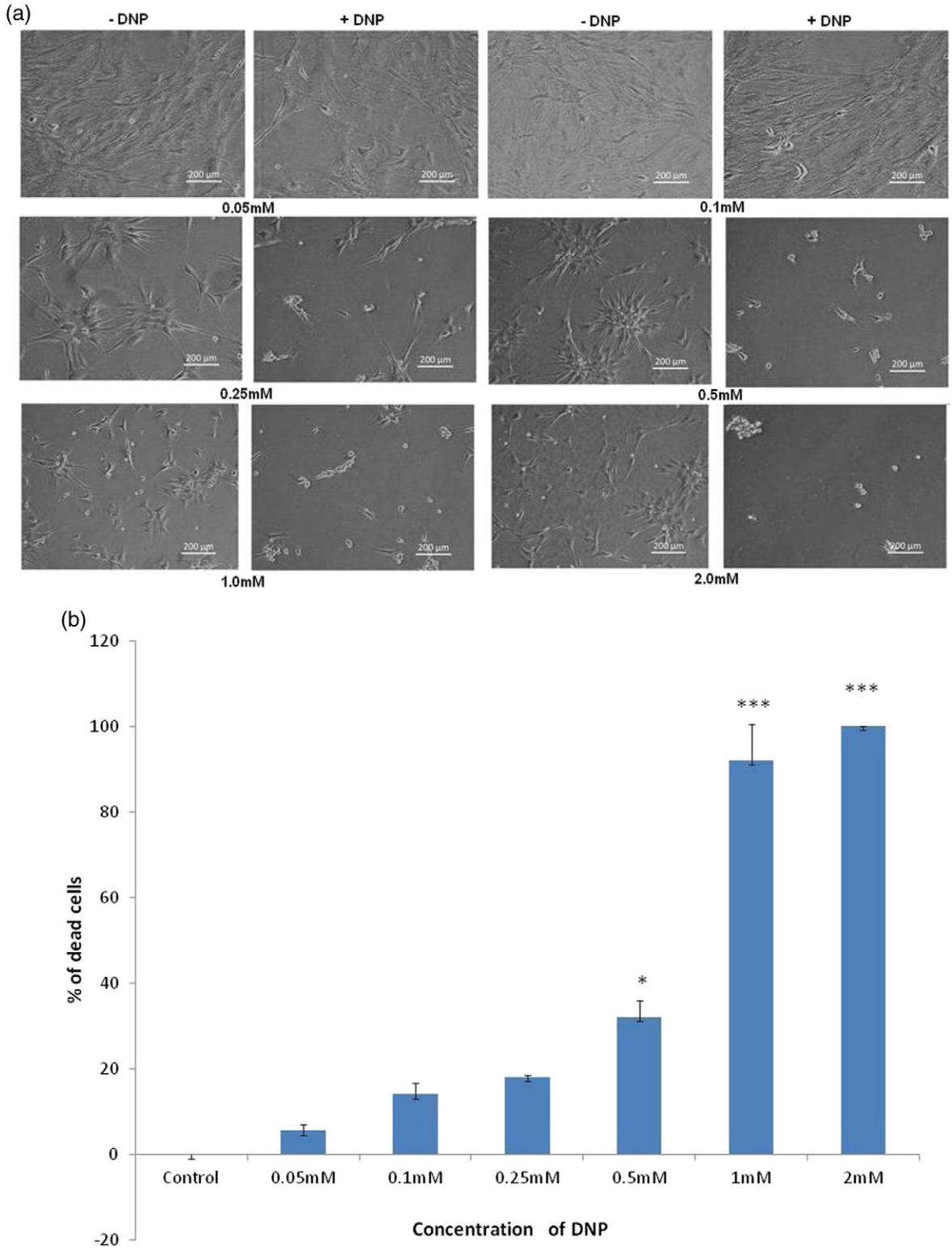


**Fig. 2.** Treatment of mesenchymal stem cells (MSCs) with different concentrations (0.05–2 mM) of DNP. (a) Morphology of MSCs showing no change at 0.05 mM and 0.1 mM concentrations. At 0.25 mM and 0.5 mM concentrations, cells were slightly shrunken while at 1 mM and 2 mM concentrations, almost all cells were dead; (b) Bar diagrams showing results of cell viability assay with trypan blue staining. No significant difference was observed in the number of dead cells between control and DNP treated MSCs at concentrations of 0.05, 0.1 and 0.25 mM; however, the number of dead cells increased significantly at 0.5 ($p < 0.05$), 1 and 2 mM ($p < 0.001$) concentrations. Data is presented as means ± standard error of means (SEM). p values <0.05 were considered statistically significant.

demonstrated that MSCs are able to survive better when exposed to limited oxygen supply that seems to be natural as these cells are found in the bone marrow where normal environment comprises low oxygen (Lavrentieva et al., 2010). Preconditioning results in the stimulation of endogenous mechanisms resulting in multiple responses including activation of specific cell surface receptors by growth factors and cytokines (Takahashi et al., 2006; Wisel et al., 2009).

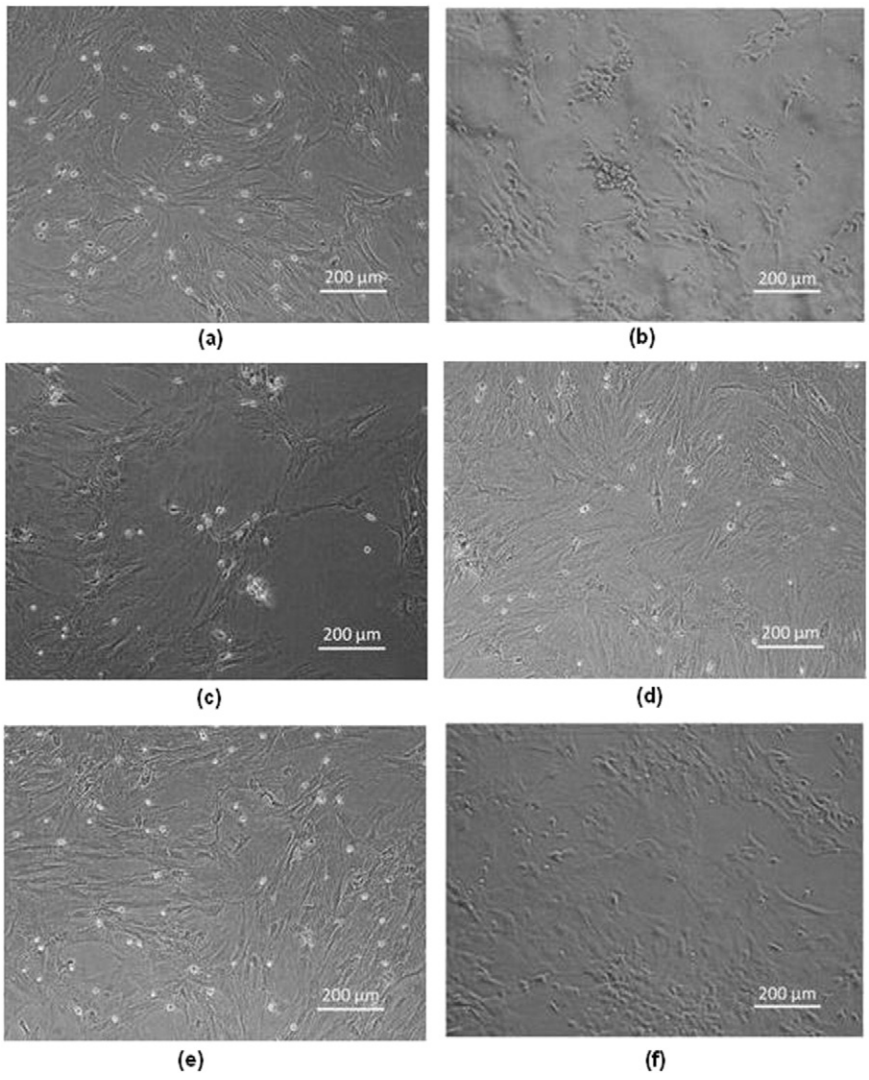


**Fig. 3.** Treatment of mesenchymal stem cells (MSCs) with 0.25 mM DNP for 20 min: (a) Control having untreated MSCs; (b) MSCs treated with 0.25 mM DNP and (c–f) MSCs treated with 0.25 mM DNP and re-oxygenated for 2, 6, 12 and 24 h respectively.

We, therefore, conducted the present study that involves a promising strategy of preconditioning the MSCs with a metabolic inhibitor that tends to deplete the energy recourses of cells to an extent that would result in an overall improved cellular efficiency. In this context, 2,4-dinitrophenol (DNP), an inhibitor of energy (ATP) production in cells, was used and its effect was analyzed on the expression levels of certain cell survival, angiogenic and cardiomyogenic factors in rat bone marrow derived MSCs at different time periods after re-oxygenation. DNP facilitates proton flux back into the mitochondrial matrix, thus, uncouples oxidative phosphorylation from ATP production (Loomis and Lipmann, 1948) and inhibits intracellular phosphorylation of glucose (Morgan et al., 1959). It has been shown to increase the uptake of glucose and D-xylose by isolated rat diaphragm by accelerating the transfer of these sugars across the muscle-cell membrane (Randle and Smith, 1958a,b). Similarly, Caldeira da Silva et al. (2008) showed that DNP could prevent the development of oxidative stress by decreasing ROS levels in mice. Recently, DNP has been proposed to drive the differentiation process of embryonic stem cells into neurons (Freitas-Correa et al, 2013). It would, therefore, be important to know the influence of DNP on the expression levels of angiogenic and cell survival factors in stem cells as this may precondition the cells for regenerative therapy where the viability of transplanted cells in an ischemic environment is of prime concern. Furthermore, genetic expression patterns of cardiomyogenic and angiogenic factors, in relationship to reduced oxidative phosphorylation, would give an insight to the roles played by these factors in case of cardiac damage due to ischemic heart diseases.

## 2. Materials and methods

### 2.1. Animals

All animal procedures were carried out in accordance with the international guidelines for the care and use of laboratory animals and approval of the local ethical committee, "Scientific Advisory Committee on Animal Care, Use and Standards". Sprague Dawley (SD) rats weighing 250–300 g were used in this study. The animals were housed in the animal facility of ICCBS, University of Karachi.

### 2.2. Isolation of mesenchymal stem cells (MSCs)

MSCs were isolated from tibia and femur of SD rats. Whole bone marrow was cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Boston, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. Cultures were maintained at 37 °C in a humidified atmosphere containing 5% $CO_2$. Non-adherent hematopoietic cells were removed from the culture. The cells were sub-cultured using 0.25% trypsin when they reached 70–

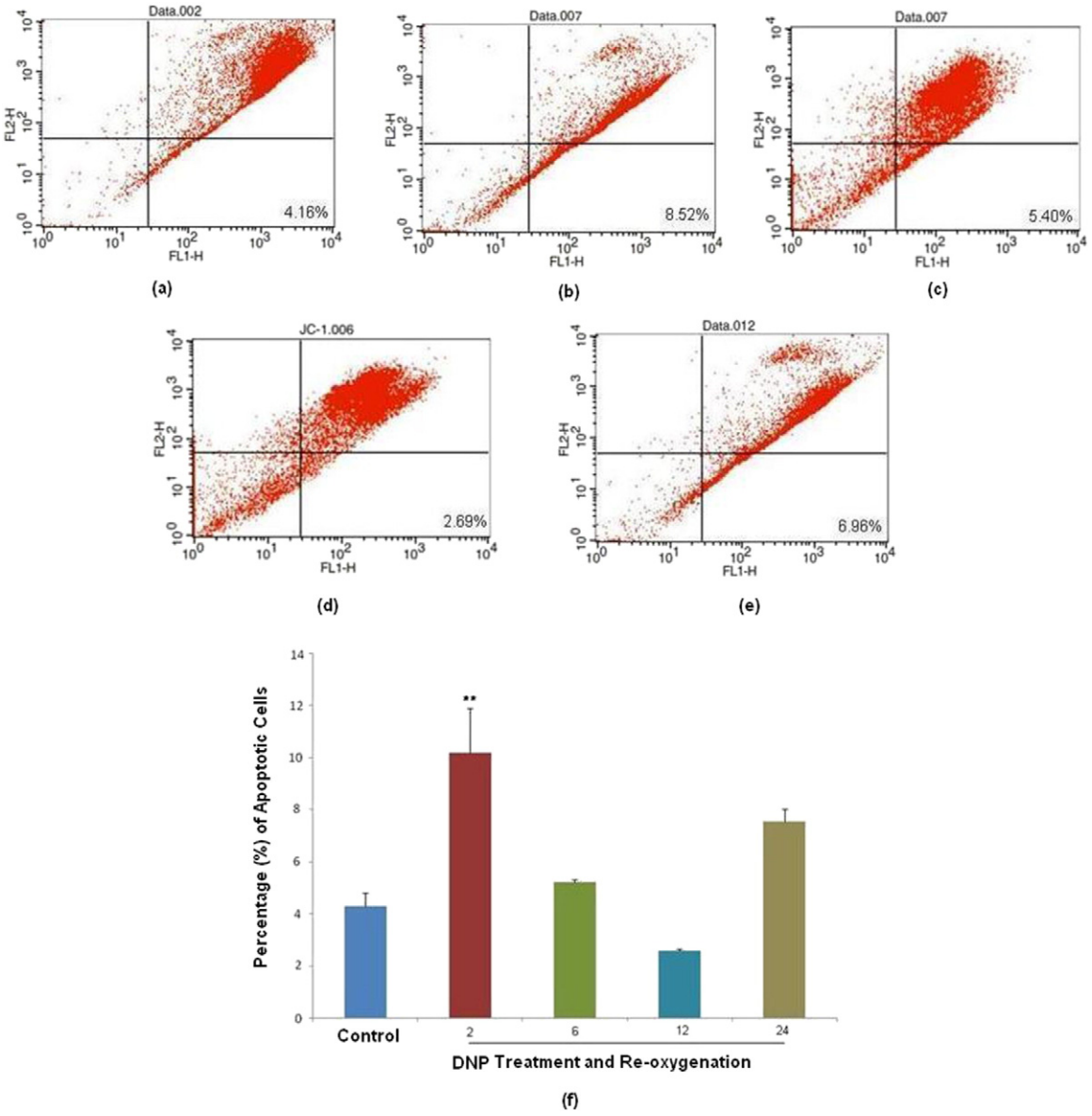


**Fig. 4.** Analysis of apoptotic MSCs after DNP treatment and re-oxygenation: (a) Untreated MSCs and MSCs treated with DNP after (b) 2, (c) 6, (d) 12 and (e) 24 h of re-oxygenation. Control and treated MSCs were labeled with JC1 stain and analyzed by flow cytometry. Apoptotic cells shift from FL2 to FL1 region; (f) Bar diagram showing number of apoptotic MSCs. MSCs after DNP treatment and 2 h re-oxygenation showed significantly higher number of apoptotic cells (**$p < 0.01$) as compared to untreated control. In contrast, there is no significant difference between untreated control and all other groups. Data is presented as means ± standard error of means (SEM). p values <0.05 were considered statistically significant.

80% confluence. Passage 1–2 bone marrow MSCs were used in all experiments.

### 2.3. Characterization of MSCs by immuno-cytochemistry

Cultured MSCs were analyzed for the presence of specific cell surface markers by immuno-staining using antibodies against stem cell growth factor receptor (c-kit or CD117), homing associated cell adhesion molecule (CD44), glycosylphosphatidylinositol-anchored glycoprotein (Thy-1 or CD90), and hematopoietic progenitor cell antigen (CD34). Cells were fixed in 4% paraformaldehyde, and blocked in phosphate-buffered saline (PBS) containing 2% bovine serum albumin (BSA), 2% normal goat serum and 0.1% Tween 20 followed by incubation with primary antibodies at 1:200 dilution in blocking solution overnight at 4 °C. This was followed by incubation with Alexa fluor 546 goat anti-mouse secondary antibody at a dilution of 1:200 for 1 h at room temperature. The cells were counter-stained with 0.5 μg/mL DAPI (4′,6-diamidino-2-phenylindole) and examined under fluorescent microscope (Nikon, Japan).

### 2.4. DNP treatment

MSCs were washed with glucose free DMEM. 2,4-Dinitrophenol (DNP) was used to induce metabolic stress chemically (Jovanović et al., 2009). The concentration and time period of DNP treatment were optimized such that the cells remain viable and the number of dead cells was limited in number. The analysis was done morphologically and by cell viability measurement using trypan blue staining. Trypan blue was diluted to 0.8 M in PBS. Cells were trypsinized and trypan blue solution was mixed at a ratio of 1:1. Cells were counted in hemocytometer. Viable cells excluded trypan blue, while dead cells stained blue due to trypan blue uptake. The optimized dose of 0.25 mM was used for 20 min throughout the experiments. DNP containing medium was replaced with high glucose DMEM. The cells

were then incubated for 2, 6, 12 and 24 h at 37 °C in a humidified atmosphere containing 5% $CO_2$ for re-oxygenation.

### 2.5. Cytotoxicity measurement (JC1 staining)

Cytotoxicity measurement of MSCs at the optimized dose of DNP was performed by JC1 staining. MSCs were cultured at a density not exceeding $1 \times 10^6$ cells/mL. Cells were washed with glucose free DMEM, treated with 0.25 mM DNP for 20 min and re-oxygenated for 2, 6, 12 and 24 h. Untreated cells were used as control. Cells were trypsinized and the dissociated cell suspension was centrifuged at 120 ×*g* for 8 min. Cell pellet was re-suspended in 0.5 mL of 10 μg/mL JC1 stain. Cells were incubated at 37 °C in humidified $CO_2$ incubator and then centrifuged at 400 ×*g* for 5 min. Cell pellet was re-suspended in PBS followed by centrifugation at 400 ×*g* for 5 min. This step was repeated two times. Finally, pellet was re-suspended in 0.5 mL PBS. Analysis was done in flow cytometer (FACS calibur, Becton Dickinson, USA).

### 2.6. Gene expression analysis

Total RNA from untreated control and DNP treated groups was isolated using SV Total RNA Isolation System (Promega, USA) according to the manufacturer's instructions. RNA concentration was measured at 260 nm. One microgram of RNA was reverse transcribed using RevertAid™ First Strand cDNA Synthesis kit (Fermentas, USA) and amplified using oligonucleotide primers. Various cardiomyogenic, angiogenic and cell survival factors were selected. Forward and reverse primers were designed accordingly using primer3 online software (http://frodo.wi.mit.edu/). Details of the genes and their primers used in this study along with their sequences, expected product sizes and annealing temperatures are listed in Table 1. Rat GAPDH gene was used as standard. The products of reverse transcription reactions were denatured for 2 min at 94 °C, followed by 30 cycles of amplification (denaturation at 94 °C for 1 min, annealing at calculated temperatures; 51–62 °C for 1 min and extension at 72 °C for 1 min) and a final extension at 72 °C for 10 min. Each PCR product was electrophoretically resolved on 1% agarose gel. Flourchem AlphaEaseFC software was used to measure the gel density of the specific primer bands. Density of each band was adjusted relative to GAPDH density in all samples.

### 2.7. Statistical analysis

Student two-tailed t test was used for comparison of two experimental groups. Multiple comparisons were done using one-way ANOVA followed by either Bonferroni's or Tukey's post-hoc test for multiple pair-wise analyses. Differences were considered significant at $p < 0.05$. All measured values are reported in means ± standard error of means (SEM).

## 3. Results and discussion

The cellular mechanisms underlying ischemia–reperfusion injury and apoptosis may involve many cellular events which include over production of oxygen-derived free radicals, cellular acidosis, and

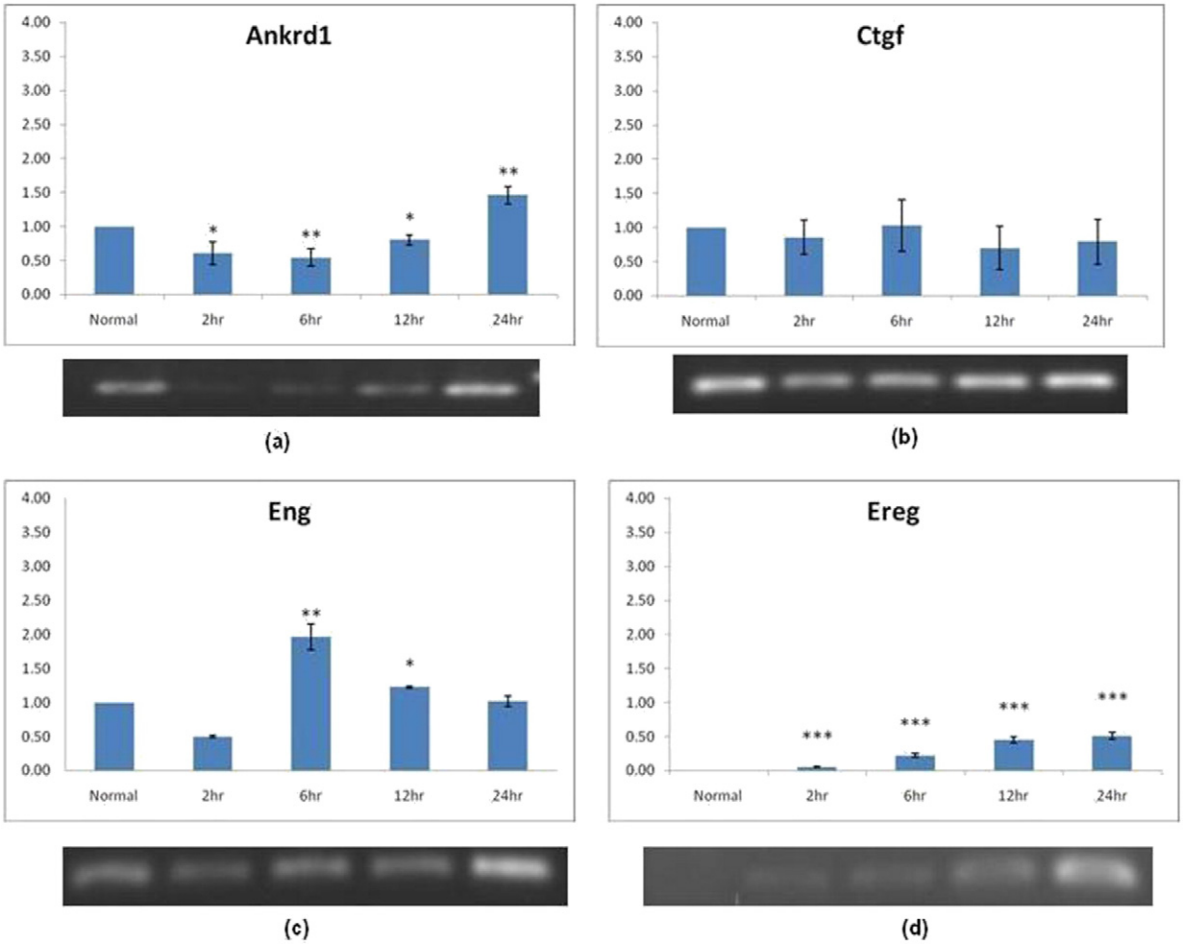


**Fig. 5.** Bar diagrams showing densitometry analyses of (a) Ankrd1, (b) Ctgf, (c) Eng, (d) Ereg, (e) Gata6, (f) Jag1, (g) Nrp1 and (h) Pgf expressed in MSCs in response to DNP treatment after 2, 6, 12 and 24 h of re-oxygenation. Y-axis shows the relative expression of each gene with respect to GAPDH expression. Expression of each gene was calculated as fold change with control value taken as 1. Each bar diagram is accompanied by agarose gel image showing gene expression analysis performed by RT-PCR for each group. Data is presented as means ± standard error of means (SEM). p values <0.05 were considered statistically significant.

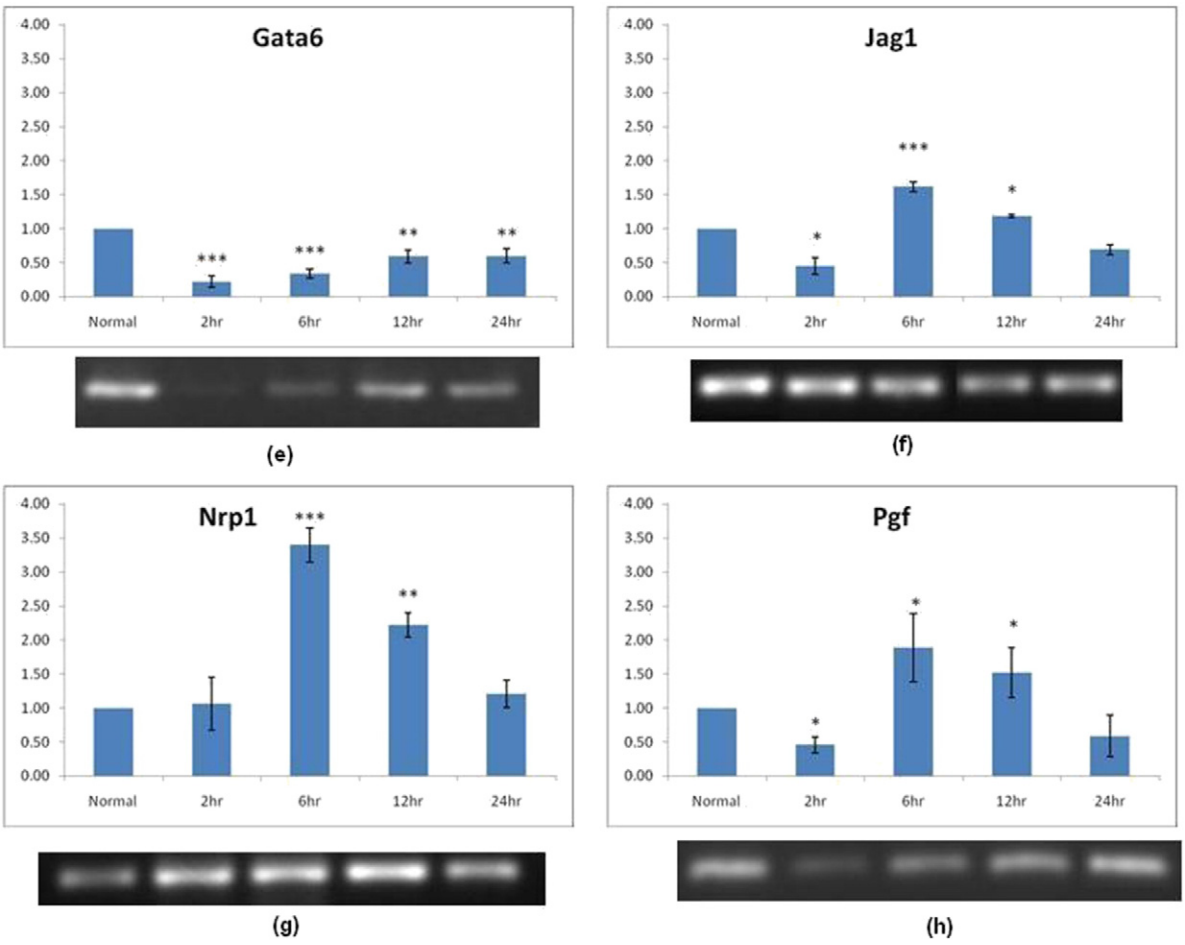


**Fig. 5** (*continued*).

inflammatory reaction (Entman et al., 1991; Thompson, 1995). Oxygen deprivation is the characteristic feature of many multi-pathophysiological processes, including tumorigenesis and organ fibrosis (Haase, 2009; Wouters and Koritzinsky, 2008). These processes result in changes in the expression levels of various specific growth factors (Crisostomo et al., 2008; Gnecchi et al., 2006; Kinnaird et al., 2004; Lee et al., 2010; Zhang et al., 2007). In the present study, we aimed to determine the pattern of change in the expression levels of different growth factors in MSCs under the influence of transient metabolic stress. Cultured MSCs under normal condition showed homogeneous population with fibroblast like morphology. Immuno-cytochemical analysis showed that the cells were positive for c-kit, CD44 and CD90 and negative for hematopoietic marker, CD34 (Fig. 1). To induce metabolic shock in MSCs, we used an optimized concentration of 0.25 mM DNP for 20 min. Morphological observation showed that at a concentration of 0.1 mM or less, no effect of DNP was observed while greater than 1 mM was found to be toxic to cells (Fig. 2a). Cell viability assay with trypan blue staining showed that there was no significant difference in the number of dead cells between control and DNP treated MSCs at concentrations of 0.05, 0.1 and 0.25 mM; however, the number of dead cells increased significantly at 0.5, 1 and 2 mM concentrations. The trend showed an increase in the number of dead cells as the concentration of DNP increased (Fig. 2b). DNP is classically known as a mitochondrial uncoupling agent (De Felice and Ferreira, 2006). It is a known metabolic inhibitor that decreases intracellular ATP production and induces energy deprivation in cells (Han et al., 1996). It is used with success to induce metabolic stress in different cell types (Brady et al., 1996; Jovanović et al., 1998; De Felice and Ferreira, 2006). For the comparison, different recovery periods (re-oxygenation) were used following metabolic stress and cells were maintained for 2, 6, 12 and 24 h at normal culture conditions. In all cases, MSCs regained their normal morphology after re-oxygenation (Fig. 3). Quantitative analyses of apoptotic cells confirmed that there is no significant difference in the number of apoptotic cells in the case of normal and treated MSCs after 6, 12 and 24 h of re-oxygenation, whereas the number of apoptotic cells was significantly high ($p < 0.01$) in the case of 2 h re-oxygenation (Fig. 4).

We selected 13 growth factors that are known to play significant roles in cell survival, angiogenesis and cardiomyogenesis. These include Ankrd1, Ctgf, Eng, Ereg, Gata6, Jag1, Kindlin3, Kdr, Myog, Nrp1, Pgf, Tbx20 and Tek. We analyzed their mRNA expressions in untreated control and DNP treated MSCs through RT-PCR. The selection of these genes was based on the criteria that MSCs having therapeutic potential to be used for cellular regeneration may have improved potential if these genes can be over-expressed. To specifically focus on enhancing the cardiac and angiogenic regeneration potential of MSCs in the degenerative tissue, we selected some cardiomyogenic and angiogenic factors. Cardiomyogenic factors are those which play important role in cardiac development while angiogenesis is the key mechanism that is also involved in the development and regeneration of cardiac cells. Two very important issues that have proved to be major barriers in cardiac regenerative therapy are the ineffective engraftment and reduced ability of these cells to survive within the infarcted myocardium. Regenerative potential of the transplanted stem cells is extremely affected by molecular processes including migration and recruitment of cells into the affected region of the heart, adhesion of the recruited cells to the injured area, and their subsequent differentiation into the desired myocardium. In addition to these three factors, the most challenging part in

**Table 2**
Fold change in the gene expression levels in normal and DNP treated MSCs after 2, 6, 12 and 24 h of re-oxygenation.

| Genes[a] | Fold change | | | | |
|---|---|---|---|---|---|
| | No treatment (control) | After DNP treatment and re-oxygenation[b] | | | |
| | | 2 h | 6 h | 12 h | 24 h |
| Ankrd1 | 1 | 0.60 ± 0.17 | 0.54 ± 0.13 | 0.80 ± 0.07 | 1.46 ± 0.13 |
| Ctgf | 1 | 0.85 ± 0.25 | 1.03 ± 0.38 | 0.70 ± 0.32 | 0.79 ± 0.33 |
| Eng | 1 | 0.50 ± 0.02 | 1.97 ± 0.19 | 1.23 ± 0.02 | 1.02 ± 0.08 |
| Ereg | 0 | 0.05 ± 0.004 | 0.22 ± 0.03 | 0.45 ± 0.04 | 0.51 ± 0.05 |
| Gata6 | 1 | 0.22 ± 0.08 | 0.34 ± 0.07 | 0.59 ± 0.09 | 0.60 ± 0.10 |
| Jag1 | 1 | 0.45 ± 0.12 | 1.62 ± 0.07 | 1.18 ± 0.02 | 0.69 ± 0.08 |
| Nrp1 | 1 | 1.06 ± 0.39 | 3.40 ± 0.25 | 2.22 ± 0.18 | 1.21 ± 0.20 |
| Pgf | 1 | 0.45 ± 0.12 | 1.88 ± 0.50 | 1.52 ± 0.37 | 0.59 ± 0.31 |

[a] Myog, Kindlin3, Kdr, Tbx20 and Tek showed no expression in normoxic and hypoxic MSCs at any time periods during re-oxygenation.
[b] Values are shown as means ± standard error of means (SEM).

the myocardial regenerative therapy is the ischemic environment of the injected cells at the infarcted region which is both nutrient and oxygen-depleted and poses a major threat to the cells damaging the overall effects of the therapy. In this milieu, over-expression of cell survival genes under the influence of anaerobic DNP could serve as an enhancer effect to the transplanted cells. In order to propose a role of MSCs in the redevelopment of cardiac wall following myocardial infarction, we propose that DNP may be a potential candidate to be used to precondition the MSCs prior to transplantation into the heart after optimized re-oxygenation periods. Recently, we have reported improved fusion of MSCs with cardiomyocytes in co-culture studies after treatment of these cells with DNP (Haneef et al., 2014). A number of molecular effects of DNP have been demonstrated that result via the regulation of key molecules within the cell. DNP is shown to be neuro-protective at low micromolar concentrations and protects against central and peripheral neuro-degeneration (da Costa et al., 2010). It promotes neuronal differentiation in a neural cell line and neurite outgrowth in primary cultured neurons, an effect that is accompanied by stimulation of cAMP signaling (Wasilewska-Sampaio et al., 2005; Sebollela et al., 2009; Freitas-Correa et al., 2013). Shavell et al. (2011) have shown that the DNP is able to induce the adhesion phenotype in fibroblasts, which is normally expressed in response to hypoxia, via a mechanism distinct from up-regulation of HIF-1α. DNP could also prevent the development of oxidative stress by decreasing ROS levels in mice (Caldeira da Silva et al., 2008). At nontoxic concentrations, DNP could stimulate energy metabolism through mechanisms which involve a mild metabolic stress response (Liu et al., 2008). DNP activates nitric oxide synthase and Akt-dependent pathways leading to mitochondrial biogenesis in mice (Cerqueira et al., 2011). Therefore, DNP can be used to precondition MSCs to enhance the regenerative potential of these cells through mechanisms that involve regulation of specific genes that play significant roles in cell survival and regeneration.

In our study, these groups of genes showed mixed pattern of expression that seemed to be dependent on the period of re-oxygenation. Fig. 5 shows the expression levels of these genes in normal and treated MSCs after 2, 6, 12 and 24 h of re-oxygenation. Fold change in the expression of these genes is outlined in Table 2. Out of these, 7 genes (Ankrd1, Ctgf, Eng, Gata6, Jag1, Nrp1 and Pgf) were expressed in the normal MSCs. The gene expression level of Ankrd1 was markedly decreased in all re-oxygenation periods except in the case of 24 h where the expression was significantly increased. Gata6 expression was markedly decreased in all re-oxygenation periods. This decrease is more pronounced in the earlier re-oxygenation periods. Ankrd1 and Gata6 are mainly involved in cardiomyogenic growth. Ankrd1 has been suggested to act as a nuclear transcription co-factor whose expression is increased during heart failure (Zolk et al., 2002) and cardiac hypertrophy (Aihara et al., 2000). It is up-regulated in response to shear stress in vitro which suggests that its expression might be associated with pathological stress in cardiomyocytes (Yoshisue et al., 2002). Gata6 belongs to the family of GATA proteins, of which six GATA factors have been identified. During embryonic and fetal mouse development, Gata6 is widely expressed in different cells and tissues (Molkentin, 2000). It is also crucial for the promotion of endothelial cell function and survival (Froese et al., 2011).

The expression levels of Pgf, Nrp1, Jag1 and Eng increased after DNP treatment showing variable pattern at different re-oxygenation periods. In most cases, we observed that there is a gradual increase in the expression levels as the time of re-oxygenation is increased and by the end of 24 h, their expression levels started to gradually decrease and would most likely reach normal values. Pgf expression was decreased after 2 h while increase in its expression was observed at 6 and 12 h of re-oxygenation. Nrp1 and Eng showed over-expression after 6 h of re-oxygenation. However, their levels began to decrease gradually; at 12 h it was still significantly high but restored to normal after 24 h of re-oxygenation. Jag1 expression was downregulated after 2 h but increased after 6 and 12 h of re-oxygenation. The level was restored to normal after 24 h of re-oxygenation. Pgf plays an important role in pathological angiogenesis and is thought to be an independent biomarker in patients with coronary artery disease. Pgf expression has been reported to be up-regulated in cardiomyocytes after hypoxia and therefore, could facilitate cardiac healing following myocardial hypoxia/ischemia (Torry et al., 2009). Nrp1 and Jag1 are angiogenic in nature and facilitate the development of new blood vessels. Angiogenesis is a crucial mechanism which should be activated when any tissue is deprived of oxygen. New blood vessels reform the disconnected bridge between blood and tissue and hence, compensate for the lesser oxygen supply to the tissues. During hypoxia, MSCs adjust their expression levels of angiogenic growth factors in order to form new blood vessels and to survive the oxygen deprived state. Eng is responsible for the growth of endothelial cells. Its expression has been shown to increase during myocardial infarction (MI). Endothelial cells are activated during hypoxia via endoglin/ALK-1/SMAD1/5 signaling in vivo and in vitro, thus regulating endothelial cell activity during adaptive cardiac angiogenesis (Tian et al., 2010). Nrp1 was shown to act as a co-receptor for VEGF protein family (Grunewald et al., 2010). It plays an important role in axon guidance, vasculogenesis, and angiogenesis (Gerhardt et al., 2004; Kawasaki et al., 1999). Jag1 is one of the ligands of notch signaling pathway that regulates development by controlling cell fate determination, cell proliferation, differentiation, and apoptosis during embryonic and postnatal stages (Bray, 2006). It has been demonstrated that hypoxia can induce Jag1 mRNA expression in the annulus fibrosus of rat disc tissue, though the possible mechanism was not explored (Hiyama et al., 2011).

Ereg did not show any expression in the normal MSCs but after DNP treatment and re-oxygenation, its expression increased gradually in the subsequent re-oxygenation periods. This shows that DNP is able to not only modulate but also induce the expression of certain genes. Ereg is a member of the epidermal growth factor (EGF) family of peptide growth factors (Riese et al., 1998). It is up-regulated in exercise and is also effective in wound healing (Draper et al., 2003; Komurasaki et al., 2002). Ereg may therefore, play an important role in the repair of damaged tissues as a result of hypoxic or ischemic insult. In the case of Ctgf, no change in the expression levels was observed after all re-oxygenation periods. Ctgf is multifunctional and its increased expression is observed during wound repair and at sites of connective tissue formation (Igarashi et al., 1993). It is highly likely that the response of Ctgf to hypoxia may be tissue dependent. Kindlin3, Myog, Tbx20, Kdr and Tek did not show any expression before or after DNP treatment and re-oxygenation.

## 4. Conclusion

It can be concluded from the present study that DNP can be used for preconditioning the stem cells in order to promote the expression of certain angiogenic, cardiomyogenic and cell survival factors. MSCs, under the influence of DNP induced metabolic stress, adjust their gene

expression levels for survival and thus can enhance their potential to regenerate the injured tissue. However, the levels of expression of these factors vary with the extent of stress and time of re-oxygenation. Recent data from different cellular therapeutic strategies currently being used for ischemic heart diseases show poor viability and lack of functional improvement of engrafted stem cells due to the harsh hypoxic microenvironment (Patel et al., 2008). It is crucial to monitor varying response of growth factors with time of re-oxygenations as a result of transient metabolic insult to MSCs so that novel strategies can be designed to improve the therapeutic potential of these stem cells to make them more beneficial against various hypoxic and ischemic diseases.

## Conflict of interest

The authors state no conflict of interest.

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